Center for Veterinary Biologics
and
National Veterinary Services Laboratories
Testing Protocol

Supplemental Assay Method for Titration of Porcine Transmissible Gastroenteritis Virus

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1. Introduction

This is an *in vitro* assay which utilizes cytopathic effect (CPE) in a cell culture system to determine viral titers of modified live porcine transmissible gastroenteritis (TGE) virus vaccines.

2. Materials

2.1 Equipment/instrumentation

2.1.1 36° ± 2°C, 5% ± 1% CO_2, high-humidity incubator[1] meeting the requirements in the current version of GDOCSOP0004

2.1.2 Water bath[2]

2.1.3 Inverted microscope[3]

2.1.4 96-well microtiter plates[4]

2.1.5 Vortex mixer[5]

2.1.6 Micropipetters: 200 µl and 1000 µl single channel;[6] 300 µlx12 channel[7]

2.2 Reagents/supplies

2.2.1 TGE Reference Virus[8]

2.2.2 Swine testicular (ST) cells free of extraneous agents as tested by the Code of Federal Regulations, Title 9 (9 CFR) 113.52[9]

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[1] Model 3158, Forma Scientific, Inc., Box 649, Marietta, OH 45750-0649 or equivalent
[2] Cat. No. 15-461-10, Fisher Scientific, Inc., 319 West Ontario, Chicago, IL 60610 or equivalent
[3] Model CK, Olympus America, Inc., 2 Corporate Center Dr., Melville, NY 11747-3157 or equivalent
[4] Costar 3596, Costar Corp., 1 Alewife Center, Cambridge, MA 02140 or equivalent
[5] Vortex-2 Genie, Model G-560, Scientific Industries, Inc., 70 Orville Dr., Bohemia, NY 11716 or equivalent
[6] Pipetman, Rainin Instrument Co., Mack Rd., Box 4026, Woburn, MA 01888 or equivalent
[7] Finnpipettes, Cat. No. NX204662D, A. Daigger Company, Inc., 199 Carpenter Ave., Wheeling, IL 60090 or equivalent
[8] Reference quantities available from the Center for Veterinary Biologics-Laboratory (CVB-L), P.O. Box 844, Ames, IA 50010 or equivalent.
[9] Provided upon request by the CVB-L or equivalent

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2.2.3 Minimum Essential Medium (MEM)

2.2.3.1 9.61 g MEM[10]

2.2.3.2 2.2 g sodium bicarbonate[11]

2.2.3.3 Q.S. to 1000 ml with deionized water, adjust pH to 6.8-6.9 with 2N hydrochloric acid (HCl).[12]

2.2.3.4 Sterilize through 0.22-µm filter.[13]

2.2.3.5 Aseptically add:

1. 10 ml L-glutamine[14]

2. 5 ml lactalbumin hydrolysate or edamin[15]

3. 100 units/ml penicillin[16]

4. 50 µg/ml gentamicin sulfate[17]

5. 100 µg/ml streptomycin[18]

6. 2.5 µg/ml amphotericin B[19]

2.2.3.6 Store at 4° ± 2°C.

2.2.4 Growth Medium

2.2.4.1 900 ml MEM

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[10] MEM with Earle's salts without sodium bicarbonate, Cat. No. 410-1500EF, Life Technologies, Inc., 8400 Helgerman Ct., Gaithersburg, MD 20884 or equivalent
[11] Cat. No. S 5761, Sigma Chemical, Inc., P.O. Box 14508, St. Louis, MO 63178 or equivalent
[12] Cat. No. 9535-01, J.T. Baker, Inc., 222 Red School Ln., Phillipsburg, NJ 08865 or equivalent
[13] Cat. No. 12122, Gelman Sciences, 600 S. Wagner Rd., Ann Arbor, MI 48106 or equivalent
[14] L-glutamine-200mM (100X), liquid, Cat. No. 320-503PE, Life Technologies, Inc., or equivalent
[15] Edamin S, Cat. No. 59102, Sheffield Products, P.O. Box 630, Norwick, NY 13815 or equivalent
[16] Penicillin solution, Schering Laboratories, 2000-T Galloping Hill Rd., Kenilworth, NJ 07033 or equivalent
[17] Gentocin solution, Schering Laboratories or equivalent
[18] Streptomycin solution, Schering Laboratories or equivalent
[19] Fungizone, E.R. Squibb & Sons, Inc., 1 Squibb Dr., Cranberry, NJ 08512 or equivalent

2.2.4.2 Aseptically add 100 ml fetal bovine serum (FBS), heat inactivated at 56° ± 2°C for 30 min ± 5 min.

2.2.4.3 Store at 4° ± 2°C.

2.2.5 Dilution Medium

2.2.5.1 98 ml of MEM

2.2.5.2 2 ml of FBS

2.2.5.3 Store at 4° ± 2°C

2.2.6 12x75-mm polystyrene tubes[20]

2.2.7 Self-refilling, 2-ml repetitive syringe[21]

2.2.8 10-ml syringe[22] and needle[23]

3. Preparation for the test

3.1 Personnel qualifications/training

Personnel must have experience in the basis of cell-culture techniques, virus titration assays, and in the principles of aseptic technique.

3.2 Preparation of equipment/instrumentation

3.2.1 Set the water bath at 36° ± 2°C

3.3 Preparation of reagents/controls

3.3.1 Two days prior to test performance

3.3.1.1 Seed 96-well microtiter plates with ST cells, in Growth Medium, at a cell count that will produce a monolayer after 2 days of

[20] Falcon 2058, Becton Dickinson Labware, Becton Dickinson & Co., 2 Bridgewater Lane, Lincoln Park, NJ 07035 or equivalent
[21] Wheaton, Cat. No. 13-689-50C, Fisher Scientific, Inc. or equivalent
[22] Luer-Lok®, Cat. No. 309604, Becton Dickinson Labware or equivalent
[23] 20 ga, 1.5 in, Cat. No. 250107, Becton Dickinson Labware or equivalent

incubation at 36° ± 2°C. These become the ST plates. Growth Medium is changed if excess acidity of the medium is observed or cells are not confluent 2 days after incubation.

3.3.2 On day of test performance

3.3.2.1 Rapidly thaw a vial of TGE Reference Virus in 36° ± 2°C water bath.

3.3.2.2 Reference Virus titration
Make 5 serial 10-fold dilutions of Reference Virus as follows:

1. Place 1.8 ml of Dilution Medium into 5, 12x75-mm polystyrene tubes labeled 10^{-1} to 10^{-5} respectively using a repetitive syringe.

2. Transfer 200 µl of Reference Virus to the 10^{-1} tube; mix by vortexing. Discard pipette tip.

3. Transfer 200 µl from the 10^{-1} tube to the 10^{-2} tube; mix by vortexing. Discard pipette tip.

4. Repeat **3.3.2.2** to the remaining tubes, transferring 200 µl from previous dilution to the next dilution.

3.4 Preparation of the test vaccine (on day of test performance)

3.4.1 Rehydrate Test Vaccine according to manufacturer’s instructions with a syringe and needle. Porcine rotavirus, if present in the test vaccine, is not usually blocked with specific antisera due to its poor growth on ST cells in the absence of proteolytic enzymes.

3.4.2 Prepare serial 10-fold dilutions of Test Vaccine. Serial 10-fold dilutions may be made as follows:

1. Place 1.8 ml of Diluent Medium into labeled tubes using repetitive syringe.

2. Pipette 200 µl of Test Vaccine to the 10^{-1} tube, mix by vortexing. Discard pipette tip.

3. Repeat **step 3.4.2.2** to the remaining tubes transferring 200 µl from previous dilution to the next dilution. Continue as needed (10^{-2} to 10^{-5}).

4. Performance of the test

4.1 Decant the Growth Media from ST plates.

4.2 Pipette 200 µl/well from each Test Vaccine tube to 5 wells on an ST plate.

4.3 Pipette 200 µl/well of each Reference Virus titration tube to 5 wells on an ST plate.

4.4 Maintain 5 or more wells as uninoculated cell-culture controls.

4.5 Incubate the ST plates undisturbed at 36° ± 2°C in the CO_2 incubator for 96 hr ± 12 hr.

4.6 96 hr ± 12 hr inoculation, examine the wells with an inverted microscope. The CPE of TGE is visible as cellular death in the cell monolayer where the cells have been destroyed by the virus.

4.6.1 Record the number of wells/dilution, showing any characteristic CPE of TGE for each Test Vaccine and Reference Virus Titration.

4.6.2 The 50% tissue culture infective dose ($TCID_{50}$) of the Test Vaccine and Reference Virus Titration are

calculated using the Spearman-Kärber method as modified by Finney. The titers are expressed as $\log_{10}$ $TCID_{50}$ per dose.

Example:

10^{-1} dilution of vaccine = 5/5 wells CPE positive
10^{-2} dilution of vaccine = 5/5 wells CPE positive
10^{-3} dilution of vaccine = 2/5 wells CPE positive
10^{-4} dilution of vaccine = 0/5 wells CPE positive

Spearman-Kärber calculation of total CPE positive wells

(12), using 5 wells =1.9

$\log_{10}$ of reciprocal of dilution counted (10^{-1}) =1.0

$\log_{10}$ of reciprocal of dose factor:

$$\frac{\text{0.1 ml inoculum}}{\text{1-ml dose}} = \frac{1}{10} \qquad =1.0$$

Total=3.9

Titer of the vaccine is $10^{3.9}$ $TCID_{50}$ per 1-ml dose.

5. Interpretation of the test results

5.1 The test is invalid if visible contamination is observed.

5.2 The test is invalid if CPE is observed in any of the control wells.

5.3 For a valid assay, the Reference Virus Titration must fall within plus or minus 2 standard deviations (± 2 SD) of its mean titer, as established from a minimum of 10 previously determined titers.

5.4 If the validity requirements are not met, then the assay is considered a NO TEST and can be retested without prejudice.

5.5 If the validity requirements are met and the titer of the vaccine is greater than or equal to the titer contained in the filed outline of production for the product under test, the product is considered satisfactory.

5.6 If the validity requirements are met but the titer of the Test Vaccine is lower than the required minimum, it must be retested according to 9 CFR 113.8.

6. Report of test results

6.1 Record all test results on the test record.

7. References

7.1 Cottral, G.E., (Ed.), 1978. Manual of standardized methods for veterinary microbiology. Comstock Publishing Associates, a division of Cornell University Press/Ithaca and London, pg 731.

7.2 Finney, D.J. 1978. Statistical methods in biological assay. Griffin, London. 3rd edition, pg 508.

8. Changes

8.1 This document was rewritten to meet the current NVSL/CVB QA requirements, to clarify practices currently in use in the CVB-L, and to provide additional detail. No significant changes were made from the previous protocol.